

EXHIBIT G

Nitroglycerin Therapy Is as Efficacious as Standard Estrogen Replacement Therapy (Premarin) in Prevention of Oophorectomy-Induced Bone Loss: A Human Pilot Clinical Study

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ABSTRACT

Nitric oxide (NO) is known to affect bone metabolism. Previous animal studies have shown that NO donor therapy can prevent ovariectomy (OVX)-induced as well as corticosteroid-induced bone loss. Therefore, we have carried out a 1-year human, randomized, controlled pilot clinical study to assess the efficacy of nitroglycerin (NG) in the prevention of estrogen-deficiency-induced bone loss in women. We observed that NG ointment, when applied to the skin once a day (within 4 weeks of undergoing oophorectomy), mimicked estrogen replacement therapy in prevention of bone loss. The primary outcome of bone mineral density (BMD) was not different in the two groups at the end of 1 year. Urinary N-telopeptide levels were significantly decreased after administration of either estrogen or NG. Although estrogen decreased serum osteocalcin and bone-specific alkaline phosphatase levels, NG therapy significantly increased these two markers of bone formation. Further, it was revealed that for up to 1 year, these doses of NG did not result in tachyphylaxis. This study showed for the first time that NG is as effective as estrogen in preventing bone loss in these surgically induced menopausal women. Additionally, the dose of NG used in this study was three to four times less than that generally used to affect cardiovascular homeostasis. Although in this randomized clinical study only a small number of patients was examined, data are encouraging. If these data hold true in large randomized, controlled clinical trials, then NG could emerge as an efficacious, cost-effective, affordable, safe, and convenient form of therapy (especially as an alternative therapy to hormone-replacement therapy [HRT]) for prevention of postmenopausal bone loss. (*J Bone Miner Res* 2000;15:2240–2244)

Key words: bone mineral density, osteoporosis, osteocalcin, nitric oxide synthase, menopause

INTRODUCTION

NITRIC OXIDE (NO) is a short-lived free radical involved in several biological processes as a bioregulator and as a second messenger. It inhibits osteoclastic bone resorption *in vitro*^(1–4) and regulates bone remodeling.^(5,6) Our recent *in vivo* studies in rats showed that treatment with the NO donor nitroglycerin (NG) alleviates ovariectomy (OVX), as well as corticosteroid-induced bone losses.^(7,8) NO at low

doses not only promotes growth of osteoblasts⁽⁹⁾ and enhances mineralization in culture,⁽¹⁰⁾ but it also partly mediates the protective effect of estrogen against bone loss.⁽⁷⁾ We have also shown that a single daily dose of NG applied to the skin is more effective than multiple daily applications,⁽¹¹⁾ suggesting practicality for potential clinical use of this economical treatment.

Sex-steroid hormones (e.g., estradiol) influence constitutive production of NO by bone cells. When estradiol is low,

NO production by the constitutive NO synthase (c-NOS) enzyme is reduced. Because NO increases osteoblastic activity and decreases osteoclastic activity, resulting in a positive bone balance, the enhancement of the local production of NO by estrogen in a controlled manner (i.e., via c-NOS) is likely to produce positive bone homeostasis. NO is synthesized in many cells, including osteoblastic cells, by the NOS enzyme(s).^(12,13) Recent evidence suggests that NO has important roles in the regulation of both osteoblast and osteoclast functions.⁽¹⁻¹⁴⁾ Inhibitory effects of NO on bone resorption have been well documented in various *in vitro* studies involving isolated rat osteoclasts, mouse calvarial metacarpal, and *in vitro* bone explant assays.^(1-6,12-14) Mechanical strains also are known to increase NO production by bone,^(15,16) and this may be involved, in part, in exercise-induced beneficial effects on bone strength.

MATERIALS AND METHODS

Study design

A randomized, open-labeled pilot study was carried out in young oophorectomized women (age, 36–45 years) to compare the efficacy of NG with that of estrogen (Premarin) for the prevention of oophorectomy-induced early postmenopausal bone loss. The study was not designed to compare these two drugs against a placebo, because the beneficial effects of estrogen in preventing postmenopausal bone loss are well established. We obtained Food and Drug Administration (FDA; IND 52,000) and Institutional Review Board approvals for the use of NG in humans and conducted this pilot clinical study at The University of Texas Medical Branch at Galveston (UTMB). This was a small, but randomized, 12-month pilot study ($n = 8$ patients per group). The efficacy of percutaneous NG therapy (15 mg/day) was compared with standard daily estrogen replacement therapy (0.625 mg Premarin, administered orally). Therapies were started between 3–4 weeks after surgery.

Study protocol

Sixteen premenopausal women who had undergone hysterectomy and bilateral salpingo-oophorectomy and who were not on any medication liable to affect calcium metabolism were counseled and recruited into the study. Patients were age-matched and randomized after surgery to receive either estrogen or NG. Because of potential high incidences of postmenopausal symptoms as a consequence of acute surgically induced estrogen withdrawal, this was a difficult group to recruit. The menopausal statuses of these patients were confirmed by follicle-stimulating hormone (FSH) levels (>40 mIU/ml) and estradiol levels (<25 pg/ml). Patients with medical disorders such as diabetes, hypertension, liver, and renal disease and patients with an absolute contraindication to estrogen replacement therapy (e.g., deep vein thrombosis, pulmonary embolism, or a history of estrogen-dependent cancers such as breast or endometrial cancer) were not included in the study. Patients with a history of migraine headaches also were excluded, because NG is likely to aggravate this disorder. Bone mineral den-

sity (BMD) was assessed in the lumbar spine and hip by dual-energy X-ray absorptiometry (DXA) scanning (QDR 1000) at the beginning of the study, and again at 6 months and 12 months. All subjects had a normal baseline BMD ($\text{BMD} \pm 1.0$ SD relative to young normal females).

Medications

Patients were randomized to receive either Premarin, 0.625 mg/day (standard hormone-replacement therapy [HRT] for prevention of postmenopausal symptoms and bone loss), or NG ointment (a strip of ointment containing ~15 mg of active NG, applied to the skin once daily [NG ointment 1-in. strip of USP 2% ointment; Fougere, Melville, NY, U.S.A.]). This dose was selected on the basis of data obtained from our dose-response studies in rats (in rats optimum dose was ~0.2 mg/kg/day).^(7,8,11)

All patients received 1.0 g of calcium and 400 IU of vitamin D supplementation per day starting immediately after surgery. A combination therapy group (i.e., estrogen plus NG) was not implemented in this study because none of our animal data showed any additional beneficial effect on BMD with a concomitant administration of estrogen and NG.^(7,17) Our previous data also suggested that the effects of estrogen on bone are mediated via the NO/cyclic guanosine monophosphate (cGMP) pathway,⁽⁷⁾ and this further emphasized the unlikely utility of coadministering these two drugs. As noted previously, no placebo control group was included in the design of this pilot study. This was a result of ethical reasons because the efficacy of estrogen treatment and the likelihood of losing bone rapidly if left untreated are well known for these patients. Follow-ups on these patients were done at 3, 6, and 12 months. During outpatient visits, we examined the patients for adverse effects (postmenopausal symptoms and adverse drug reactions) and recorded their blood pressures (BP). These assays were carried out at the Endocrine Sciences Laboratory, Minneapolis, MN, U.S.A.

Statistics

Baseline BMD data were analyzed with analysis of variance (ANOVA; $p < 0.05$). Changes of BMD and biomarkers within each group were examined using ANOVA and paired *t*-test. The comparisons between the two groups were made using a repeated measures ANOVA ($p < 0.05$; baseline, 6 months, and 12 months) and two-sample *t*-tests with Bonferroni correction ($p < 0.025$). Data were expressed as means \pm SEM.

RESULTS

Outcome measures in this study included DXA scanning, 24-h urine collections, and blood sampling for biochemical markers of bone turnover (serum osteocalcin, bone-specific serum alkaline phosphatase, and urinary cross-linked N-telopeptide of type I collagen [NTx]:creatinine) at baseline, 6 months, and 1 year. No significant adverse effects or changes in BP were observed for the subjects during and

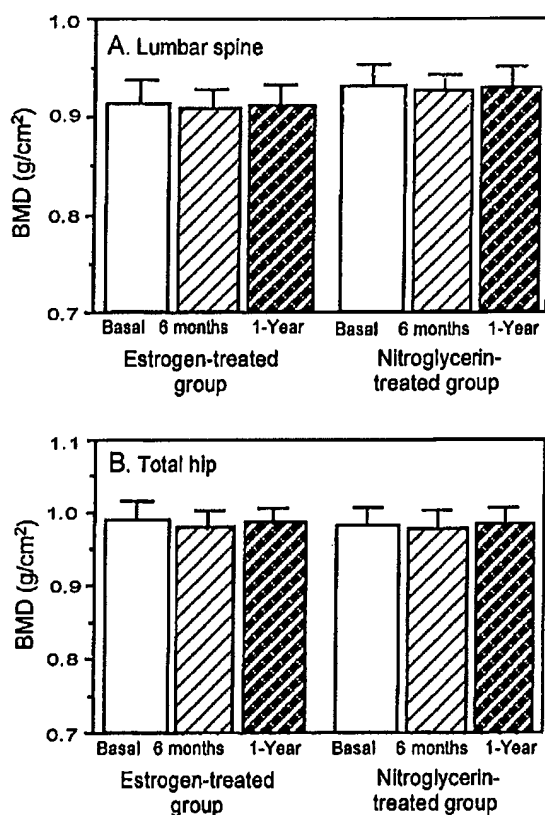


FIG. 1. Mean BMD (g/cm²) (A) in lumbar spine and (B) in total hip in estrogen (Premarin, 0.625 mg/day) and NG-treated (15 mg of NG per day) groups of oophorectomized women ($n = 7$ per group; mean \pm SEM). At baseline (open columns), at 6-months (hatched columns), and at 12 months (filled columns) of treatment (no statistical differences were observed between any treatment groups).

after therapy. However, not surprisingly, the incidence of hot flashes recorded was higher in the NG-treated group; a total of 20 episodes were experienced by the estrogen-treated group compared with 42 episodes in the NG-treated group. One patient from each treatment group dropped out during the first month of therapy. Therefore, data are presented on seven patients per group.

BMD

Figure 1 illustrates the BMD data in the lumbar spine and the total hip at baseline, 6 months, and 12 months in these two groups of women. Our hypothesis was that the effects of NG on BMD in the spine and hip are not different from that of estrogen in the prevention of early menopausal bone loss (NS). Therefore, we did not expect the primary endpoint of the two treatment groups (i.e., BMD) to differ. In these patients, no significant changes in BMD in the lumbar spine or hip were observed after 6 months or 12 months of

treatment, in comparison with baseline values in both groups of women. In addition, no changes in BMD were seen between groups or within each group during the study period. In these early menopausal women, the efficacy of NG in preservation of BMD was equivalent to that of standard doses of estrogen (i.e., both drugs were "equally effective" in preventing bone loss).

Biochemical markers

Figure 2 illustrates the biochemical markers of bone turnover in these oophorectomized patients (basal, 6 and 12 months of treatment). NG significantly increased serum osteocalcin (5.3 ± 0.9 ng/ml vs. 8.1 ± 0.8 ng/ml, 53% increase; normal range, 3–14 ng/ml) as well as serum bone-specific alkaline phosphatase levels (33.8 ± 4.4 IU/liter vs. 43.0 ± 3.1 IU/liter; 27% increase), whereas estrogen therapy decreased both these variables. Both estrogen and NG significantly decreased urinary NTx levels (normal range, 5–65 nM Bone Collagen Equivalent (BCE)/nM creatinine; 25–40% decrease). These findings are identical to those we previously observed with our animal studies.^(8,11,17) Although changes in bone biochemical markers were not extraordinarily high, in the long term, an ~30% decrease in urinary NTx with a ~30% increase in osteocalcin and bone-specific alkaline phosphatase should have a positive effect on bone balance and consequent increase of BMD and the bone strength in the long term. Bone-specific alkaline phosphatase levels in serum closely paralleled the trends with serum osteocalcin suggesting that the increase of bone formation markers observed in this study was coming from the bone. A similar finding was previously reported in rats.⁽¹¹⁾

DISCUSSION

In this 1-year randomized controlled human pilot study, we observed that NG ointment applied to the skin once a day (within 4 weeks of undergoing oophorectomy) mimicked estrogen replacement therapy and prevented bone loss. If untreated, these women would normally experience an accelerated bone loss (in the range of 4–8% during the first year after OVX) secondary to rapid loss of estrogen after the surgery.^(18,19) However, the primary outcome BMD was not different in the two groups at the end of 1 year or from the respective baseline.

This pilot study showed that NG is as effective as estrogen in preventing bone loss in these surgically induced menopausal women. Further, it was revealed that these doses of NG for up to 1 year do not result in tachyphylaxis.⁽²⁰⁾ Similar data are available for rats for up to 6 months (Sunil Wimalawansa, unpublished data, 1999). Additionally, the dose of NG used in the human study (three to four times less than that generally used for cardiovascular treatments)⁽²¹⁾ did not cause any significant adverse effects. Higher incidence of hot flashes was seen in the NG-treated group in comparison to estrogen-treated women. Whether this is the natural background incidence of hot flashes or the number of episodes were actually increased because of the

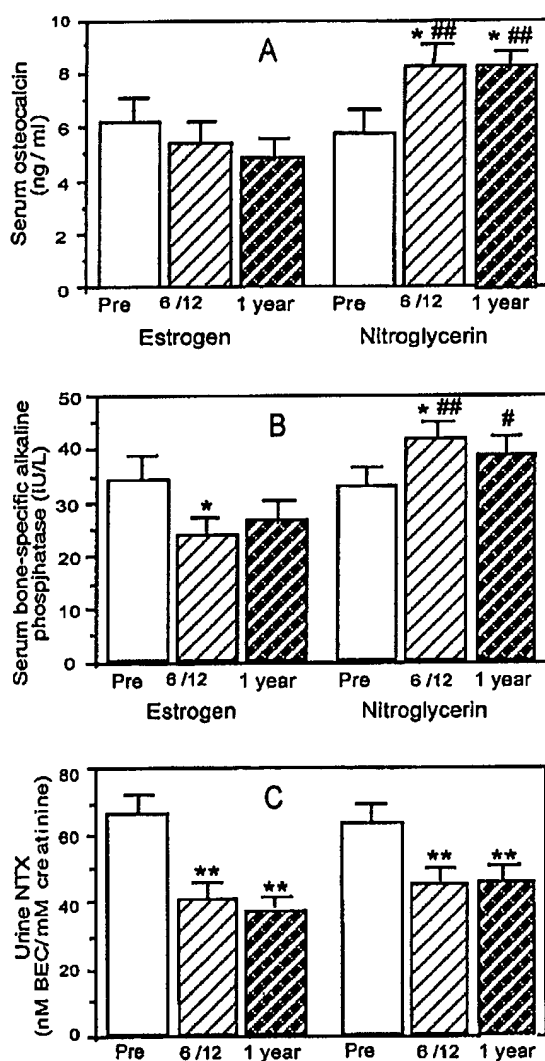


FIG. 2. (A) Serum osteocalcin (ng/ml), (B) serum bone-specific alkaline phosphatase (IU/liter; markers of bone formation), and (C) urinary NTx (nM BEC/mM creatinine) levels (marker of bone resorption) in oophorectomized women receiving Premarin (0.625 mg/day) or NG (15 mg/day; $n = 7$ per group). Mean \pm SEM data at baseline (open columns), at 6-months (hatched columns), and at 12 months (filled columns) of treatment are presented. Six months and 12 months data compared with the baseline data using ANOVA and paired t -test and indicated by * $p < 0.05$ and ** $p < 0.025$ (with Bonferroni correction). Between group comparisons were performed using two sample t -test # $p < 0.05$ and Bonferroni correction ## $p < 0.025$.

use of NG (because NG is a vasodilator) is not clear as we did not include a placebo-treated group. There was no difference in the incidence of headaches between the two

groups. Therefore, not only is NG efficacious and cost-effective, but it is also a safe and convenient form of therapy for the prevention of bone loss. However, a larger randomized control clinical study is needed to confirm these findings.

Although both drugs equally decreased urinary NTx levels, only NG increased serum osteocalcin and bone-specific alkaline phosphatase levels. This suggests the possibility of an increase in bone formation in response to NG therapy and corroborates our *in vitro* data and *in vivo* animal data.^(7,11,17) Treatment of these women with calcium and vitamin D from the time of surgery may have dampened the basal levels of biomarkers observed in this study.⁽²²⁾ However, the data indicate that 15 mg NG applied transdermally once a day prevented expected bone loss and had a positive impact on markers of bone turnover⁽²³⁾ in these oophorectomized women. However, the doses of NG used in this study reflect the doses we used successfully in the prevention of bone loss in ovariectomized rats. Because the optimal doses in the rats (nonremodeling species) may not necessarily be the same in the humans, we suggest that future studies are necessary to identify both the optimum dose and the frequency of administration of NG to get the best results in humans.

The incidence of osteoporosis is rising because of an increase in the elderly population throughout the world.^(18,24) However, therapeutic options are still limited because available therapeutic agents have adverse effects and also are increasing in cost.⁽²⁵⁾ There are a number of effective inhibitors of osteoclastic bone resorption: estrogen, selective estrogen receptor modulators, calcitonin, and bisphosphonates.^(24,25) However, these drugs are expensive, and the long-term consequences of some of these drugs are unclear.^(18,24) Also, none of these agents have any significant effects on osteoblastic bone formation (i.e., no demonstrable anabolic effect on bone).^(18,25) On the other hand, NG had been used over the past few decades and its safety profile is well established.^(20,26) NG is a cost-effective drug and is very safe at the doses used in this study. Considering the changes in the health care system in the United States, it is paramount not only that these new agents are effective and devoid of major short- and long-term side effects, but also that they be affordable. Given the magnitude of problems linked to osteoporosis, the only cost-effective approach is prevention.^(18,27) Clearly, an alternative treatment that is safer, more effective, and affordable is needed for osteoporosis.

Taken together, these *in vivo* human data and previous rat studies⁽¹⁻¹⁶⁾ show that NG prevents bone loss associated with estrogen deficiency. In addition, *in vitro* studies suggest that at low doses, NO donors promote the growth of osteoblasts and suppress osteoclastic bone resorption.^(1-4,9,10) Furthermore, serum NO levels are decreased in patients with amenorrhea with osteopenia,⁽²⁸⁾ and estrogen replacement therapy increased circulating NO levels.⁽²⁹⁾ Rosselli and colleagues provided further *in vivo* evidence of enhancement of the release of NO in the presence of estrogen.⁽³⁰⁾ Given these findings, we previously hypothesized that the protective effects of estrogen against bone loss are mediated partly through the NO/cGMP pathway.⁽⁷⁾ We fur-

ther believe that these protective mechanisms may indicate that NO donor therapies have the potential to emerge as a new group of potent, effective, and economical agents in preventing (and possibly treating) menopausal bone loss, particularly as an alternative therapy for estrogen.

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